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USE OF CHOLESTEROL-LOWERING AGENTS TO INFLUENCE SIGNAL  
TRANSDUCTION PROCESSES IN THE CELL MEMBRANE AND IN THE PROPHYLAXIS  
OR TREATMENT OF PRION-ASSOCIATED DISEASES OR ALZHEIMER'S DISEASE

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**Abstract**

**The invention relates to the use of cholesterol-lowering agents in the prophylaxis or treatment of diseases based on conformational change of prions and Alzheimer's disease as well as for influencing signal transduction processes in the cell membrane.**

## FOR INFORMATION ONLY

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This invention concerns the use of cholesterol-reducing agents for prophylaxis or treatment of diseases that involve a conformational change of prions, or of Alzheimer's disease.

For most molecular biologists who are concerned with membrane proteins the function of lipids lies mainly in their ability to serve as solvent for proteins (Singer & Nicolson, *Science* 175 (1972), 720-731). However, this is not their only role. The different types of lipids are arranged not only in a fluid double layer, but they are also asymmetrically distributed over the exoplasmic and cytoplasmic membrane regions (Bretscher and Raff, *Nature*, 258 (1975), 43-49; Roelofsen & Op den Kamp, *Plasma Membrane Phospholipid Asymmetry and its Maintenance: The Human Erythrocyte as a Model* 1-7-46 (1994)). In addition, it was found that the lipids are also organized in a certain way and thus carry out more regulation tasks than was previously known (Glaser, *Curr. Op. Struct. Biol.* 3 (1993), 475-481, Thomas et al., *J. Cell Biol.* 125 (1994), 195-802, Kusumi & Sako, *Curr. Opin. Cell Bio.* 8 (1996), 566-574). It now turned out that a lateral organization of lipids results through the combination of sphingolipids and cholesterol into shifting floes or rafts, to which proteins can specifically attach within the double layer. The existence of such sphingolipid-cholesterol rafts leads to a fundamentally different evaluation of the membrane organization and allows new insights into the function of cell membranes.

The models shown in Figure 1B were developed on the basis of research into such sphingolipid-cholesterol rafts. The starting point is that the sphingolipid head groups, which occupy larger areas of the plane of the exoplasmic part of the membrane than the hydrocarbon chains of the lipids in the membrane layer, allow the development of intermediate spaces that become filled by cholesterol molecules, which function as, so to say, spacers (Figure 1B). A dense joining together of these sphingolipid-cholesterol clusters to the exoplasmic part of the membrane allows them to function as the overall arrangement within the membrane double layer. It is important to establish here that the sphingolipids normally have a long fatty acid ( $C_{20}$ - $C_{26}$ ) which is attached to the sphingosine base via an amide bond, where, because of its length, the fatty acid can join to the cytoplasmic part of the double layer of the membrane. Since cholesterol is present in both membrane layers, it is also possible that the molecule will act as a spacer in the cytoplasmic part of the membrane and, in doing so, fill the intermediate spaces between fatty acids present there (Figure 1B). The new findings on the organization of sphingolipids and cholesterol in the cell membrane have now led to the recognition that in this there could also be a basis for treatment or prevention of diseases that involve a change of conformation of prion proteins or even Alzheimer's disease. Such diseases are still not treatable, for the most part even detecting them is very difficult, and in most cases absolute certainty can be obtained only by an autopsy after the death of the patient. From that standpoint there is a compelling need for a possibility of treating such diseases, at least in a suspected case, or of preventing their development.

For this reason the task of this invention was to make available a possibility of being able to have a positive effect on diseases like Alzheimer's disease or other diseases in which a change of proteins on sphingolipid-cholesterol rafts takes place.

This task was solved in accordance with the invention through the use of cholesterol lowering agents for prophylaxis or treatment of diseases that stem from a change of conformation of prions, or of Alzheimer's disease. All agents that lower the cholesterol level in the blood and are used or can be used for this purpose for prevention of other diseases, above all arteriosclerosis and heart attack, can be used as cholesterol lowering agents. Examples of cholesterol lowering agents include the active agent lovastatin (Mevinacor, Mevinolin, Monacolin-K, MK-803) as well as other drugs for treating hypercholesterolemia such as pravastatin-sodium, simvastatin, bezafibrate, clofibrate, etofylline clofibrate, xenofibrate, gemfibrozyl, etofibrate, colestipol-HCl, colestyramine, xantinol nicotinate, icositol nicotinate, probucol and the like. Lovastatin inhibits cholesterol biosynthesis on the basis of mevalonic acid. It is already being used as a drug to treat hypercholesterolemia, where it is administered in doses up to 20 mg/day. The dosages of cholesterol lowering agents in accordance with the invention are known or can easily be determined by the specialist.

Another possible manner and way of lowering the cholesterol level is to affect the regulation of cholesterol metabolism. The distribution of cholesterol on sphingolipid-cholesterol rafts is much higher than its distribution in areas in which there are no rafts. In the endoplasmic reticulum, where the cellular cholesterol content is perceived and regulated, there are practically no rafts. Sphingolipid-cholesterol rafts do not flow back from the Golgi apparatus to the endoplasmic reticulum. The lowering of the cholesterol level first affects the cholesterol not contained in rafts, which leads to less cholesterol flowing back to the endoplasmic reticulum. By reducing, for example, the sphingolipid content, it would then be possible to simultaneously lower the cholesterol synthesis in the cell, since more cholesterol can flow back to the endoplasmic reticulum. This can occur, for example, through sphingolipid synthesis inhibitors.

The disease scrapie, which occurs in sheep, is seen above all at the present time as a disease that derives from a change of conformation of prion proteins. Also, this invention may be important for the BSE problem, if it is confirmed that the triggering factor for the ultimate fatal disease is also a change of conformation of prion proteins and that this disease of cows can be transferred to humans. Therefore, treatment of Creutzfeld-Jacob disease would also be an object of this invention.

The scrapie prion protein  $\text{PrP}^{\text{Sc}}$  is the only known component of a transmissible prion that is known up to now. It is derived from a protein  $\text{PrP}^{\text{C}}$  that is normally anchored to glycosyl phosphatidyl inositol (GPI) and is expressed in neurons, where the prion protein  $\text{PrP}^{\text{Sc}}$ , which is protease resistant, arises through a conformational change of  $\text{PrP}^{\text{C}}$ . Presumably, this change of

conformation takes place on sphingolipid cholesterol rafts. The change of conformation appears to be dependent on the GPI anchoring, since chimeric proteins that contain a characteristic transmembrane domain are not subject to the conformational change. PrP<sup>C</sup> is insoluble in Triton X-100 at 4°C during the conformational change, and a depletion of cellular cholesterol hinders the formation of PrP<sup>Sc</sup>. Interestingly, PrP<sup>C</sup> can be transferred into the cell via clathrin-coated vesicles by means of endocytosis, presumably due to bonding to a still unknown transmembrane protein with a so-called coated pit signal. This binding possibly keeps the PrP<sup>C</sup> at a distance from the rafts, where the still unelucidated PrP<sup>C</sup> - PrP<sup>Sc</sup> transformation appears to take place.

One of the important characteristics of the pathogenesis of Alzheimer's disease is progressive cerebral accumulation of amyloid  $\beta$ -peptide (A $\beta$ ), a proteolytic cleavage product of an amyloid precursor protein (APP). Newly synthesized APP is directed into the axons in the neurons and then carried further to the dendrites by transcytosis. During the intercellular transport APP is subject to a number of cleavages, in which either the amyloid fragment A $\beta$  or a non-amyloid, secreted form APP<sub>sec</sub> (secreted) is released. In the cleavage to APP<sub>sec</sub> by  $\alpha$ -secretase ( $\alpha$ -cleavage) an 8 kD transmembrane fragment remains in the cell membrane. The cleavage of APP to A $\beta$  takes place in two steps. First a 10 kD fragment of APP is produced through the so-called  $\beta$ -cleavage and is then cleaved again within the transmembrane domain ( $\gamma$ -cleavage), which results in A $\beta$ . Like PrP<sup>C</sup>, a part of the APP in neurons is also insoluble in Triton X-100, a property that the GPI anchored proteins and transmembrane proteins that bind to sphingolipid rafts also have. Where exactly the A $\beta$  production takes place is not clear, but recently obtained results indicate that A $\beta$  complexes to a lipid in sphingolipid cholesterol rafts, the GM1 ganglioside, which is found in the earliest disease manifestations in the brain. Interestingly, a small part of APP is also found in detergent-insoluble, glycolipid-enriched complexes, the so-called DIGs (Brown & Rose, *J. Cell*, 68 (1992), 533-544; Parton & Simons, *Science*, 269 (1995), 1398-1399). Possibly APP is fixed into the rafts by binding to GM1 and proteolysis may possibly take place in the sphingolipid-cholesterol microdomains, so that A $\beta$  that is bound to GM1 results. The A $\beta$  peptide is localized in the APP molecule exactly where it is to be expected if one assumes that this region binds to a glycosphingolipid. Another interesting connection to the sphingolipid-cholesterol rafts is the recently obtained result that A $\beta$  binds to the receptor for glycanization end products (Yan et al., *Nature*, 382 (1996), 685-691), of which it was established that they associate with DIGs and caveolae in endothelial cells (Lisanti et al., *Developm. Biol.* 6 (1995), 47-58).

It was established in the scope of this invention that the use of cholesterol-lowering agents has a positive effect on the diseases mentioned above. This is possibly due to a lowering of the number of rafts in the plasma membranes and thus a lowering of the number of possible anchoring points, at which a change of conformation of proteins then takes place.

The realization in accordance with the invention that cholesterol-lowering drugs have a positive effect on Alzheimer's disease or diseases like Creutzfeld-Jacob disease allows for the first time a treatment possibility that attacks the causes of the disease.

Moreover, through the use of cholesterol-lowering agents in accordance with the invention it appears in general possible to affect signal transduction processes in cells from outside. It was recently shown that many proteins that are found in sphingolipid-cholesterol rafts play an important role in signal transduction (Parton & Simons, *Science*, 269 (1995), 1398-1399; Anderson, *Proc. Natl. Acad. Sci. USA*, 90 (1993), 10909-10913; Lisanti et al., *Trends Cell Biol.*, 4 (1994), 231-235).

Through the development of sphingolipid-cholesterol rafts a subcompartmentalization is brought about on the cell membrane and leads to different structures developing, thus rafts of different sizes and intermediate spaces without raft structure.

Raft formation is not possible without cholesterol and, as far as that goes, many signal transduction operations are thus dependent on the presence of cholesterol. The following may be mentioned as examples of such operations: signal transduction via heterotrimer G proteins (Li et al., *J. Biol. Chem.*, 270 (1995), 15693-15701), Ras (Song et al., *J. Biol. Chem.*, 271 (1996), 9690-9697; Mineo et al., *J. Biol. Chem.*, 271 (1996), 11930-11935) and ceramides (Liu & Anderson, *J. Biol. Chem.*, 270 (1995), 27179-27185). Examples of signal transduction processes in which rafts play a role as platforms are immunoglobulin E signal processes in allergic reactions, T cell receptor signal processes, LPS endotoxin signal processes, signal processes of endothelial NO synthase, signal processes via tyrosine kinases like Lyn and Fyn, which are doubly acylated and via trimer G proteins that contain doubly acylated subunits, and transfer signals via GPI-anchored proteins.

Another object of this invention is thus the use of cholesterol-lowering agents to affect signal transduction processes at the cell membrane.

#### Description of figures

Figure 1 shows a cell membrane in cross section. A shows a detail with rafts that contain proteins bound to the exoplasmic layer of the membrane by means of their GPI anchors. B shows an enlargement of a sphingolipid-cholesterol raft.

Figure 2 shows that the removal of cholesterol inhibits the production and secretion of A $\beta$ . a shows an immunoprecipitation assay of neurons from the hippocampus, which were cultured for 4 days either in the presence of (+) or in the absence of (-) lovastatin/mevalonate. Cyclodextrin was added for 0 or 5 or 20 min (0, 20, 5). b shows a similar immunoprecipitation assay to a, where in this case CD cholesterol was added for the indicated time in minutes (0 or 15). c shows the relative A $\beta$  secretion in cells to which the cholesterol was either removed by



means of lovastatin and cyclodextrin or added, compared to untreated control cells (average of 3-11 experiments). The figures next to cyclodextrin and CD cholesterol give the time of addition in minutes.

Figure 3 shows that the removal of cholesterol reduces the accumulation of APP in DIGs. Neurons were extracted in accordance with Example 2 and centrifuged through an OptiPrep gradient, after which larger molecules and complexes (A $\beta$ -DIG) are found more at the upper end of the test tube (top) and uncomplexed, smaller molecules (A $\beta$ ) migrate downward (bottom). - depletion or + depletion refers to the cholesterol that, as described above, was removed (+) or not removed (-).

This invention is illustrated in more detail by the following examples, where the data concerning amounts and other details are exemplary and not to be understood as exhaustive.

## Examples

### Example 1

Primary cell cultures of neurons from rat hippocampus were plated and cultured by traditional methods (minimum essential medium (MEM) with 10% horse serum, 5% CO<sub>2</sub>, 36.5°C). The addition of 5 mM cytosine arabinose prevented the multiplication of non-neuronal cells. After 5-7 days 4  $\mu$ M lovastatin and 0.25 mM mevalonate were added for 4 days.

The neurons were then infected for 1 h at 37°C and 5% CO<sub>2</sub> with recombinant SFV, which codes for the human APP 695 protein, as is already known from the prior art. The cells were incubated for 2 h in lovastatin/mevalonate and then incubated for 5-20 min with 5 mM methyl- $\beta$ -cyclodextrin (Sigma) in a methionine-free labeling medium (MEM with 1/10 N<sub>2</sub> addition). The cells were labeled with 150  $\mu$ Ci [<sup>35</sup>S]-methionine for 2.5 h. Lovastatin in the presence of small amounts of mevalonate inhibits cholesterol biosynthesis. Methyl- $\beta$ -cyclodextrin removes in particular cellular cholesterol.

After the metabolic labeling the culture medium was collected and cell extracts prepared (2% NP-40, 0.2% SDS, 5 mM EDTA, with protease inhibitors as additive). The immunoprecipitates were recovered on A Sepharose (Boehringer) and analyzed on 10-20% tris-tricine polyacrylamide gels (Novex). The radioactivity was determined by means of a phosphorus imager (Molecular Dynamics). The antibodies that were used were Fd-APP against APP 695, B12/4 against the 20C terminal amino acids of APP, and B7/6 against the amino acids 1-16 of the synthetic human A $\beta$  peptide 1-40. Figure 2 shows the results of this immunoprecipitation assay. In a cells were analyzed that had been cultured with or without lovastatin ("- " or "+") and with cyclodextrin for 0, 20 or 5 min. The visible bands correspond to A $\beta$ . Here it clearly turns out that the secretion of A $\beta$  can be reduced through the removal of

cholesterol, which is brought about through the addition of lovastatin and cyclodextrin. Cholesterol was readded to some cells (CD cholesterol).

b shows a similar immunoprecipitation assay in which, however, cholesterol was again added to the cells that are represented in the two right-hand tracks. The A $\beta$  bands are also visible there.

The diagram c shows the relative A $\beta$  secretion under different experimental conditions, which were already described above.

### Example 2

Extracts of neuronal cells were prepared as in Example 1 except that they were pulse labeled for 20 min and then chased for 100 min in normal medium. Then the cells were extracted for 30 min on ice with 1% Triton X-100 in TEX (150 mM NaCl, 50 mM tris, pH 7.4, 2 mM EDTA, 2 mM DTT, 25  $\mu$ g/mL chymostatin, leupeptin, antipain, pepstatin A each). The extracts were then mixed with an equivalent amount of OptiPrep (Nycomed) and covered with a stepwise gradient of 30%, 25% and 3% OptiPrep in TEX. Then the 3 samples were centrifuged for 3 h at 4°C and 50,000 rpm, the fractions were collected and immunoprecipitated. The result is shown in Figure 3. Control cells in which cholesterol was not removed are represented under "- depletion," while the cells of "+ depletion" had been treated with lovastatin/cyclodextrin, in order to achieve a cholesterol depletion. "top" indicates the top portion of the test tube, while "bottom" indicates the bottom portion of the test tube. With this type of gradient complexes and larger molecules such as A $\beta$ -DIG do not migrate to the bottom of the test tube, but are thus found more in the tracks that are marked with "top," while the A $\beta$  molecules not associated with DIGs migrate downward. This experiment also clearly shows that the A $\beta$ -DIG association is reduced in cells treated with cholesterol-lowering agents.

### Claims

1. The use of cholesterol-lowering agents for prophylaxis or treatment of diseases that derive from a change of conformation of prions, and of Alzheimer's disease.
2. The use of cholesterol-lowering agents to affect signal transduction processes on the cell membrane.

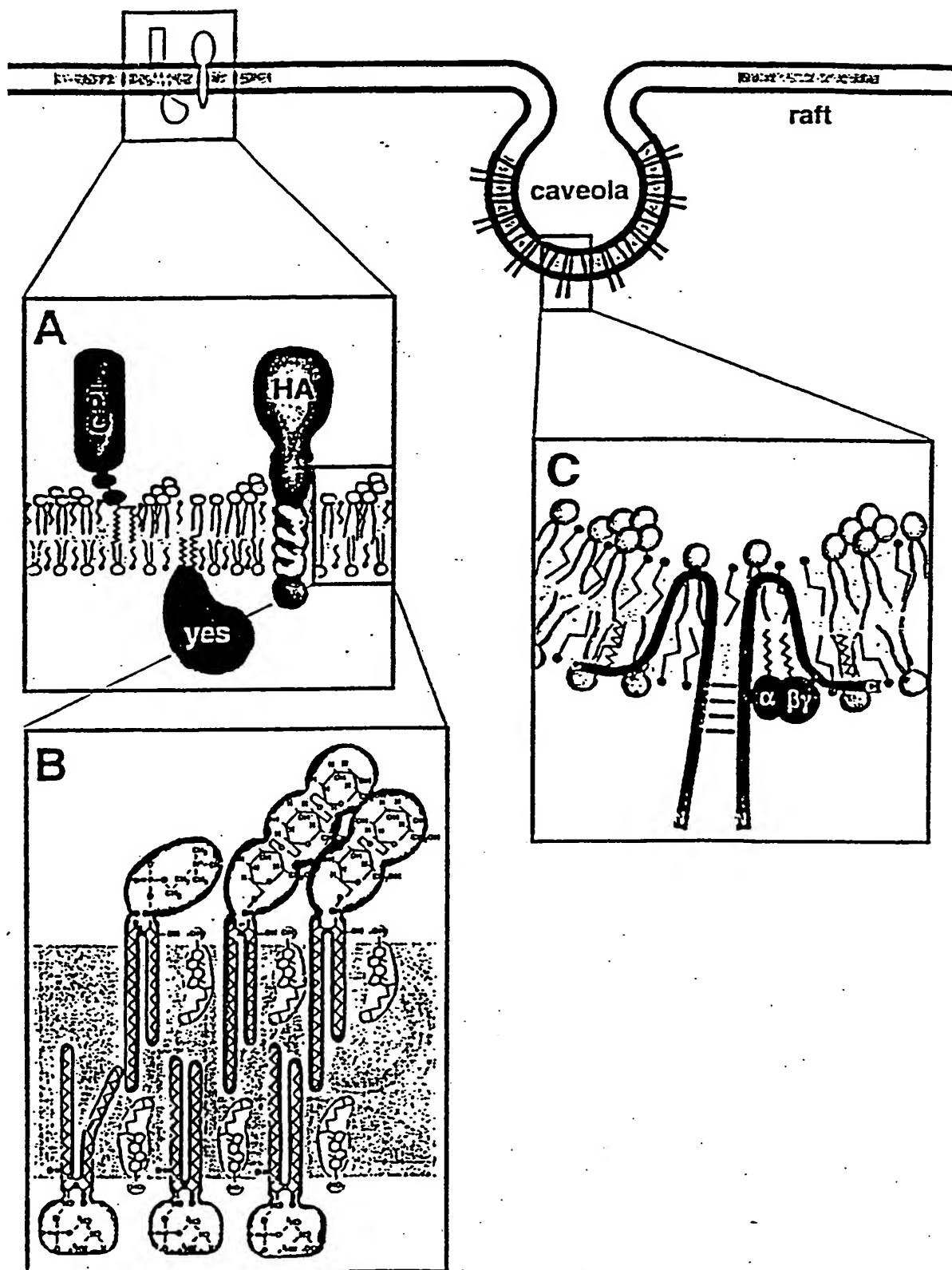


Figure 1

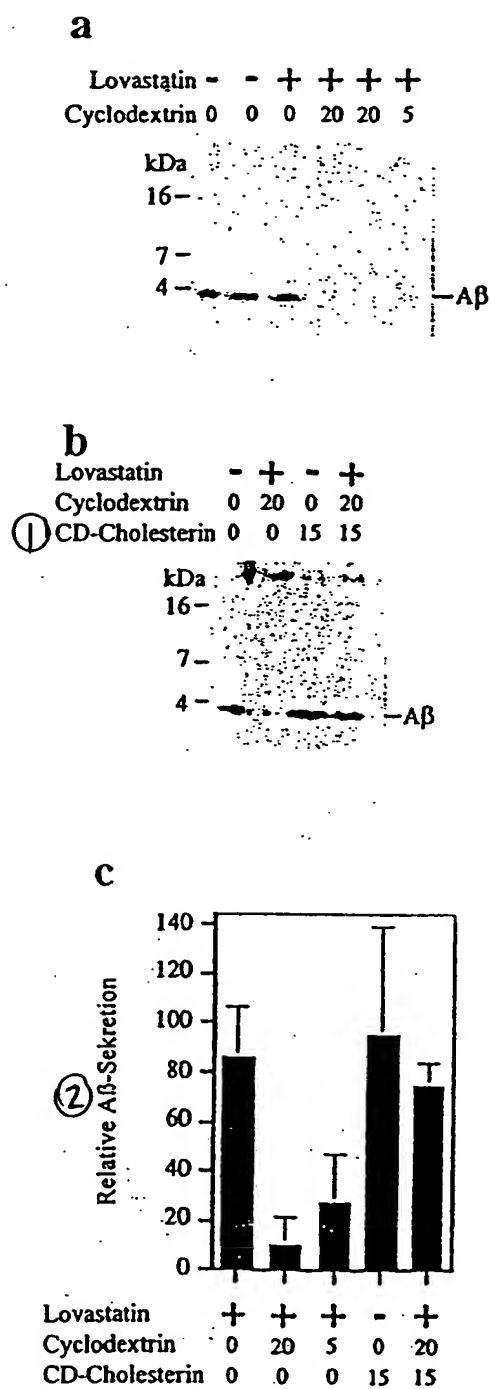


Figure 2

Key: 1 CD cholesterol  
2 Relative A $\beta$  secretion

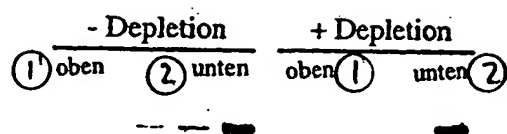


Figure 3

Key: 1 Top  
2 Bottom

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/02284

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 A61K31/715

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>KELLER AND SIMONS: "Cholesterol is Required for Surface Transport of Influenza Virus Hemagglutinin" THE JOURNAL OF CELL BIOLOGY, vol. 140, 23 March 1998, pages 1357-1367, XP002078575 see page 1358, left-hand column, paragraph 2 see page 1360, right-hand column see page 1364, left-hand column, paragraph 5 - right-hand column, paragraph 2; figures 7,8</p> <p style="text-align: center;">--- -/--</p>	1,2

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "Δ" document member of the same patent family

Date of the actual completion of the international search

24 September 1998

Date of mailing of the international search report

07/10/1998

Name and mailing address of the ISA

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 98/02284

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TARABOULOS ET AL: "Cholesterol Depletion and Modification of COOH-Terminal Sequence of the Prion Protein Inhibit Formation of the Scrapie Isoform" THE JOURNAL OF CELL BIOLOGY, vol. 129, 1995, pages 121-132, XP002078576 see page 126 see page 129, left-hand column see page 130 see page 122	1,2
X Y	WO 94 04556 A (UNIV NEW YORK) 3 March 1994 see page 1, column 14-33 see page 3, line 11-15 see page 4, line 26-30 see page 10, line 37 - page 11, line 16; claims 7-11 see page 13, line 35 - page 14, line 30; claims 13,17-19 see page 15, line 24-30 see page 19, line 15-28	2 2
X Y	US 5 569 452 A (TSRL INC.) 29 October 1996 see column 1, line 50-57; claims 1,5,7 see column 3, line 37-63	2 2
X	STANKEWICH ET AL: "Alterations in Cell Cholesterol Content Modulate Ca <sup>2</sup> -Induced Tight Junction Assembly by MDCK Cells" LIPIDS, vol. 31, no. 8, 1996, pages 817-828, XP002078577 see page 819, right-hand column; figures 1A,1C see page 820; figure 5; table 4 see page 825, left-hand column see page 826, right-hand column, line 3-5	2
X	CAMILLERI ET AL: "Beta-Cyclodextrin Interacts with the Alzheimer Amyloid Beta-A4 Peptide" FEBS LETTERS, vol. 341, 1994, pages 256-258, XP002078578 see page 256 see page 258, right-hand column, paragraph 2	1

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/02284

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BANDIERA ET AL: "Inhibitors of A-Beta Peptide Aggregation as Potential Anti-Alzheimer Agents" CURRENT MEDICINAL CHEMISTRY, vol. 4/3, 1997, pages 159-170, XP002078579 see page 159 see page 160, right-hand column - page 161, left-hand column see page 166 ---	1
X	WO 95 06470 A (MERCK & CO INC) 9 March 1995 see page 1, column 5-13; claims 1-4,6-10 ---	1
X	WO 96 19987 A (JANSSEN PHARMACEUTICA NV) 4 July 1996 see page 4, column 23-30 see page 7, column 31-35 see page 8, line 37 - page 9, line 4 see page 9, line 19-24; claims 1,2,10 ---	1
X	WO 94 02518 A (UNIV KANSAS) 3 February 1994 see page 19, paragraph 3 see page 3, paragraph 4 - page 5, paragraph 1; claims 1,30 see page 8, paragraph 4 ---	2
X	DATABASE WPI Week 7532 Derwent Publications Ltd., London, GB; AN 75-53053w XP002078580 & JP 50 035315 A (NIPPON KAYAKU KK) , 4 April 1975 see abstract & JP 50 035315 A (NIPPON KAYAKU KK) 4 April 1975 ---	2
A	WO 96 20184 A (PFIZER) 4 July 1996 see page 1, line 20-29 see page 32, line 25 - page 33, column 9 see page 34, line 13-20 -----	1



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 98/ 02284

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:

Observation: Although Claim(s) 1-2 relate to a method for treatment of the human/animal body, the search was carried out and was based on the cited effects of the compound/composition.

2. ☒ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

See Supplemental Sheet ADDITIONAL MATTER PCT/ISA/210

3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/EP 98/02284

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9404556	A	03-03-1994	AU 5081593 A CN 1087091 A	15-03-1994 25-05-1994
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